Interaction of saposin D with membranes: effect of anionic phospholipids and sphingolipids

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Saposin (Sap) D is an endolysosomal protein that, together with three other similar proteins, Sap A, Sap B and Sap C, is involved in the degradation of sphingolipids and, possibly, in the solubilization and transport of gangliosides. We found that Sap D is able to destabilize and disrupt membranes containing each of the three anionic phospholipids most abundant in the acidic endolysosomal compartment, namely lysobisphosphatidic acid (LBPA), phosphatidylinositol (PI) and phosphatidylserine (PS). The breakdown of the membranes, which occurs when the Sap D concentration on the lipid surface reaches a critical value, is a slow process that gives rise to small particles. The Sap D–particle complexes formed in an acidic milieu can be dissociated by an increase in pH, suggesting a dynamic association of Sap D with membranes. The presence of anionic phospholipids is required also for the Sap D-induced perturbation and solubilization of membranes containing a neutral sphingolipid such as ceramide or a ganglioside such as G_{M1}. At appropriate Sap D concentrations Cer and G_{M1} are solubilized as constituents of small phospholipid particles. Our findings imply that most functions of Sap D are dependent on its interaction with anionic phospholipids, which mediate the Sap D effect on other components of the membrane such as sphingolipids. On consideration of the properties of Sap D we propose that Sap D might have a role in the definition of the structure and function of membranes, such as the intra-endolysosomal membranes, that are rich in anionic phospholipids.

Key words: anionic phospholipids, membrane destabilization, membrane solubilization, saposins, sphingolipids.

INTRODUCTION

Saposin (Sap) D is a member of a family of four similar small proteins (Sap A, Sap B, Sap C and Sap D), generated in the late endosomal/lysosomal compartment from a common precursor protein, prosaposin [1–3]. One function of saposins is the promotion of sphingolipid degradation by lysosomal hydrolases. Each saposin exhibits specificity for a certain sphingolipid or group of sphingolipids. Sap A has been proposed to be involved in the degradation of galactosylceramide, since a mouse strain lacking Sap A developed a late-onset form of globoid cell leucodystrophy with storage of galactosylceramide [4]. Sap B promotes the degradation of sulphatide, as indicated by its accumulation in metachromatic-leucodistrophy patients lacking Sap B, but with normal arylsulphatase A activity [5,6]. Sap C plays an essential role in the regulation of glucosylceramide catabolism, acting as the physiological activator of glucosylceramidase [7]; moreover Sap C is able to activate the degradation of galactosylceramide in living cells [8]. Sap D has been proposed to favour the hydrolysis of ceramide (Cer) [9,10], but the absence of a specific genetic deficiency leaves unanswered the question about its physiological role.

Although saposins are involved in sphingolipid degradation, not all their properties can be assigned to this action. In fact, other functions have been suggested, such as the solubilization and transport of gangliosides and cerebrosides. Actually, it has been reported that saposins and prosaposin bind and solubilize gangliosides, forming stable complexes and promoting their transfer from donor to acceptor membranes [11]. The formation of water-soluble complexes between saposins and neutral sphingolipids such as cerebrosides has also been described [12]. Moreover, it has been recently hypothesized that the interaction of the Sap D domain of prosaposin with sphingomyelin is required for the lysosomal transport of prosaposin [13].

Conversely, we found that Sap C and D poorly interact with sphingolipids, while exhibiting a very high affinity towards anionic phospholipid-containing membranes at the low pH values characteristic of the acidic endolysosomal compartment [14–17]. At pH values lower than 5.5, a dramatic increase in Sap C and Sap D hydrophobicity leads to their spontaneous binding to anionic phospholipid-containing membranes, causing a dramatic perturbation of the lipid surfaces [15]. Moreover, high concentrations of Sap D solubilize anionic phospholipid-containing vesicles, while Sap C aggregates and possibly fuses them [18].

A key role of anionic phospholipids in promoting the activity of Sap C, the saposin required for the enzymic degradation of glucosylceramide, has been demonstrated [19,20]. In fact, we showed that the mode of action of Sap C involves as the first step its binding to anionic phospholipid-containing membranes. Upon association, Sap C destabilizes the membrane and promotes the association of glucosylceramidase, allowing the contact between the enzyme and its substrate, glucosylceramide, also present in the lipid surface [14].

Our past findings on the properties of Sap D prompted us to hypothesize that the interaction with anionic phospholipids might underpin its physiological activity. The late endosomes/lysosomes, where saposins are localized, contain internal membranes where anionic phospholipids can reach very high concentrations in a mosaic of structural and functional domains [21,22]. The anionic phospholipids most abundant in these organelles are lysobisphosphatidic acid (LBPA), phosphatidylserine (PS)

Abbreviations used: Chol, cholesterol; Sap, saposin; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; LBPA, lysobisphosphatidic acid; Cer, ceramide; MLVs, multilamellar vesicles; LUVs, large unilamellar vesicles.

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and phosphatidylinositol (PI) [21,22]. LBPA has been found exclusively in the internal membranes of multivesicular late endosomes/lysosomes, and it likely plays an important role in the transport of proteins and lipids.

To shed light on the mechanism of action of Sap D and on its role in lipid metabolism, we have now analysed in detail the impact of Sap D on membranes containing the anionic phospholipids present in the internal endolysosomal membranes (LBPA, PI and PS). A neutral sphingolipid such as Cer or a ganglioside such as G_{M1} have been also included in the lipid surfaces in order to determine the Sap D effects on sphingolipid-containing membranes, either in the presence or in the absence of anionic phospholipids.

EXPERIMENTAL

Materials

Phosphatidylcholine (PC) from egg yolk, PS from bovine brain, G_{M1} from porcine brain and oleoyl-LBPA were from Avanti Polar Lipids, Inc. (Alabaster, AL, U.S.A.). PI from bovine liver and cholesterol (Chol) were from Sigma. Cer from bovine brain was from Alexis Biochemicals, San Diego, CA, U.S.A. L-α-[*dipalmitoyl*-1-^{14}C]Dipalmitoyl PC (80–120 mCi/mmol) was from PerkinElmer Life Sciences (Boston, MA, U.S.A.). [^{3}H]Cer was prepared by hydrogenation of Cer using ^{3}H [Nycomed Amersham plc (now Amersham Health, Little Chalfont, Bucks., U.K.)] performed [^{3}H]reduction and purification of the final product]. [^{3}H]G_{M1} was prepared by labelling the ganglioside in the terminal galactose moiety [23]. The fluorescent dye Calcein was from Molecular Probes (Eugene, OR, U.S.A.). All other chemicals were of the purest available grade.

Sap D preparation

Sap D was purified from spleens of patients with Type 1 Gaucher's disease following a previously reported procedure [15]; it consisted essentially of heat treatment of a water homogenate followed by ion-exchange chromatography on DEAE-Sephacel, gel filtration FPLC on a Superdex 75 HR 10/30 column (Amersham Biosciences, Little Chalfont, Bucks., U.K.) and reverse-phase HPLC on a protein C_4 column (Vydac, Hesperia, CA, U.S.A.). The purity of the final preparation of Sap D was verified by N-terminal sequence analysis, SDS/PAGE and electrospray MS [17].

Vesicle preparation

Multilamellar vesicles (MLVs) were prepared by mixing appropriate amounts of lipids dissolved in chloroform and evaporating the solvent under nitrogen. PC was supplemented with a trace amount of [^{14}C]PC to a specific radioactivity of about 60 d.p.s./nmol. Cer and G_{M1} were supplemented with trace amounts of [^{3}H]Cer and [^{3}H]G_{M1} respectively. The specific activities of both sphingolipids were about 500 d.p.s./nmol. The dry lipids were dispersed by vortex mixing in 2 mM L-histidine/2 mM Tes/150 mM NaCl/1 mM EDTA, pH 7.4. The suspension was submitted to 10 cycles of freezing and thawing and then blended by a vortex mixer for 3–5 min. The vesicle concentration was determined by radioactivity measurements.

Leakage assay

Leakage of liposome contents was monitored by the release of Calcein trapped inside the vesicles [24]. Large unilamellar vesicles (LUVs) for leakage experiments were prepared by hydrating dried films of lipids in 60 mM Calcein, pH 7.4, followed by ten cycles of freeze–thawing. The resulting multilamellar vesicles were passed 21 times through two stacked 0.1-μm-diameter-pore polycarbonate membranes in a Liposofast-Miniextruder (Avestin, Ottawa, Canada) to convert multilamellar into unilamellar vesicles [25]. Free Calcein was separated from the dye-containing LUVs by chromatography on a Sephadex G-75 column.

Upon addition of Sap D, the release of Calcein to the external medium was followed by the increase in fluorescence caused by Calcein dilution and the consequent relief of self-quenching (excitation 470 nm, emission 520 nm); 100 % leakage was established by lysing the vesicles with 0.3 % (v/v) Triton X-100. Leakage of liposomes was carried out at 37 °C and monitored with a Fluoromax 2 spectrofluorimeter equipped with a constant-temperature cell holder and stirrer (Spex Industries Inc., Edison, NJ, U.S.A.).

Gel permeation

Mixtures of Sap D and labelled MLVs in buffer A (10 mM acetate/150 mM NaCl/1 mM EDTA, pH 4.5) were incubated for different periods of time at 37 °C and then adjusted to pH 7.4 by the addition of an appropriate amount of 50 mM NaOH. The mixtures were then applied to Sepharose CL-4B columns (1 cm × 22 cm) pre-equilibrated and eluted at room temperature with buffer B (10 mM Tris/HCl/150 mM NaCl/1 mM EDTA, pH 7.4). The flow rate was 0.25 ml/min. Fractions (0.4 ml each) were collected.

In specified experiments, a gel-permeation Superose 6HR 10/30 prepacked column (Amersham Biosciences) attached to a FPLC system was utilized. The column was pre-equilibrated and eluted either with buffer A or with buffer B. The flow rate was 0.4 ml/min. Fractions (0.4 ml each) were collected.

The lipid distribution was determined by measuring radioactivity of the fractions. When two labelled lipids were present, double-isotope counting conditions were adopted.

Reversibility of the Sap D binding to membranes

To assess the formation and dissociation of Sap D–lipid complexes, the fractions from the Superose 6HR 10/30 column (see above) were concentrated by transferring them to Microcon-YM-3 centrifugal filter devices (molecular mass cut-off 3 kDa) and centrifuging at 13 000 ***g*** until all the liquid except 100 μl had passed through the filter. The presence of Sap D in each retentate was tested by SDS/PAGE (see below).

SDS/PAGE

SDS/PAGE was performed with 15 %-(w/v)-acrylamide separating gels and 4.5 % stacking gels [26]. After electrophoresis, Sap D was revealed with the PlusOne™ Protein Silver Staining Kit (Amersham Biosciences).

RESULTS

Sap D-induced perturbation of membranes

We have previously shown that Sap D spontaneously binds to phospholipid membranes in acidic environments. The depth of its insertion and the consequent membrane destabilization is regulated by the presence of anionic phospholipids [17,18]. Since in the late endosomal/lysosomal acidic compartment, where

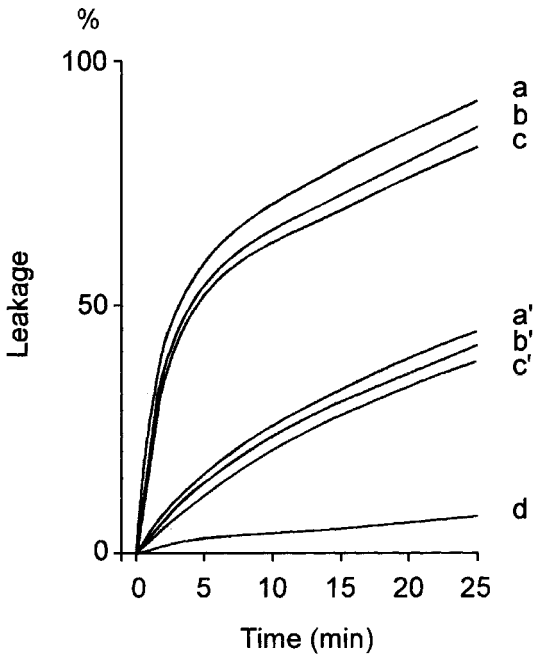

Figure 1 Effect of anionic phospholipids on the Sap D-induced leakage of vesicles

Time course of the Sap D-induced release of Calcein entrapped in LUVs composed of PC/Chol/anionic phospholipid (50:25:25 molar ratio) (a, b and c) or PC/Chol/anionic phospholipid (65:25:10) (a′, b′ and c′). The anionic phospholipids were LBPA (a and a′) or PI (b and b′) or PS (c and c′). Also, the leakage from LUVs composed of PC/Chol, 75:25 (d), was assessed. The saposin was injected into a stirred cuvette thermostatically controlled at 37 °C, containing a 1 ml suspension of liposomes (75 μM total lipid) in buffer A, pH 4.5. The lipid/saposin molar ratio was 1000:1. The experiments when repeated at least three times gave similar results.

Sap D is localized, the most abundant anionic phospholipids are LBPA, PI and PS, we compared their individual effect on the Sap D-induced destabilization by assessing the release of Calcein encapsulated into LUVs. As shown in Figure 1, Sap D, at a lipid/saposin molar ratio of 1000:1 and at pH 4.5, is able to destabilize membranes that contain each of the three anionic phospholipids, whereas in their absence the amount of released dye decreases to less than 5%. The leakage activity of Sap D was directly related to the percentage of anionic phospholipids present on the lipid surface, as indicated by the increased release of Calcein when their percentage was increased from 10 up to 25%. No leakage from the anionic phospholipid-containing LUVs was observed at pH 7.0 (results not shown).

The three anionic phopholipids had similar capacity to promote the destabilizing activity of Sap D at low pH values; after 25 min, about 80–85% of the Calcein was released from LUVs containing 25% of either PS or PI or LBPA. It is thus evident that, in an acidic environment mimicking that of the endosomal/lysosomal system, each of the three anionic phospholipids is able to promote the Sap D-induced destabilization of membranes.

Kinetics of the Sap D-induced disruption of membranes

It was previously shown that the impact of Sap D on the membrane structure is modulated by the saposin concentration on the lipid surface; at lipid/saposin molar ratios higher than 100:1, Sap D perturbs PS-containing large vesicles without affecting their size, whereas at lower ratios Sap D breaks down the membrane, giving rise to small particles enriched in PS [18]. To follow the kinetics of the disruption of the membranes, mixtures of Sap D with anionic phospholipid-containing MLVs, at a lipid/saposin molar ratio of 25:1, were incubated for various time periods at pH 4.5 and then adjusted to pH 7 to stop the membrane transformation. The analysis of the samples by gel permeation on Sepharose CL-4B columns showed that MLVs containing PS or PI or LBPA were eluted as a single peak at the excluded volume (maximum at fraction 20) (Figure 2). After 10 min of incubation with Sap D, part of the lipid was eluted as a broad shoulder shifted towards lower molecular masses. The formation of smaller particles increased with time. After 30 min, about 50–65% of MLVs were transformed, and, after 2 h of incubation, most of the lipids were converted into smaller particles that were eluted in broad peaks centred at fractions 33–37 (Figure 2). No further change of the elution patterns occurred after a 4 h incubation. At the equilibrium (2 h), a slightly higher percentage (about 85%) of the LBPA- than of the PS- or PI-containing MLVs (70–75%) were transformed. It is thus apparent that the Sap D-induced breakdown of membranes is a slow process (it takes more than 30 min under the present conditions), promoted by each of the three anionic phospholipids, LBPA being slightly more effective than PS or PI.

Reversibility of Sap D binding to membranes

At low pH values, Sap D forms strong complexes with the small anionic phospholipid-containing particles [18]. In order to investigate whether a change of pH might dissociate Sap D from the membranes, PS-containing MLVs were first incubated with Sap D at pH 4.5. Subsequently, half of the sample was analysed by gel permeation at pH 4.5 (Figure 3A), and the other half was adjusted to pH 7.4 and analysed at this pH (Figure 3B). A FPLC Superose 6HR 10/30 column, that, under our experimental conditions, can clearly distinguish between free and membrane-bound Sap D, was used to analyse the samples. On this column MLVs were eluted in the excluded volume at fraction 21. After incubation with Sap D at a lipid/saposin molar ratio of 12.5:1, the MLVs were completely transformed into smaller particles eluted in a peak centred at fraction 33. This result confirms a previous finding that, at a lipid/saposin molar ratio of 12.5:1, Sap D is able to convert almost all the large vesicles into small particles [18]. The presence of Sap D in the fractions was monitored by measuring the absorbance of the fractions and by electrophoresis. When the analysis was performed at pH 7.4, a major absorbance peak was observed at fractions 45 and 46. Such a peak was not detected after chromatography at pH 4.5, suggesting that the saposin was bound to the lipids at this pH. This result was confirmed by electrophoresis; Sap D was found in fractions 32–34 after elution at pH 4.5 (inset to Figure 3A) and in fractions 45 and 46 after elution at pH 7.4 (inset in Figure 3B). Thus an increase of pH results in the detachment of most of the saposin from the lipid surface, indicating that the binding of Sap D is, at least in part, reversible, and can be modulated by the pH of the environment.

Sap D-induced perturbation and disruption of sphingolipid-containing membranes

It has been found that Sap D activates the enzymic hydrolysis of Cer *in vitro* and *in vivo* [9,10]. To investigate whether this activation was related to the membrane-perturbing properties of Sap D, the leakage of Calcein from Cer-containing LUVs was assessed at a lipid/Sap D ratio of 1000:1, a condition that does not cause the disruption of the membranes [18]. Figure 4

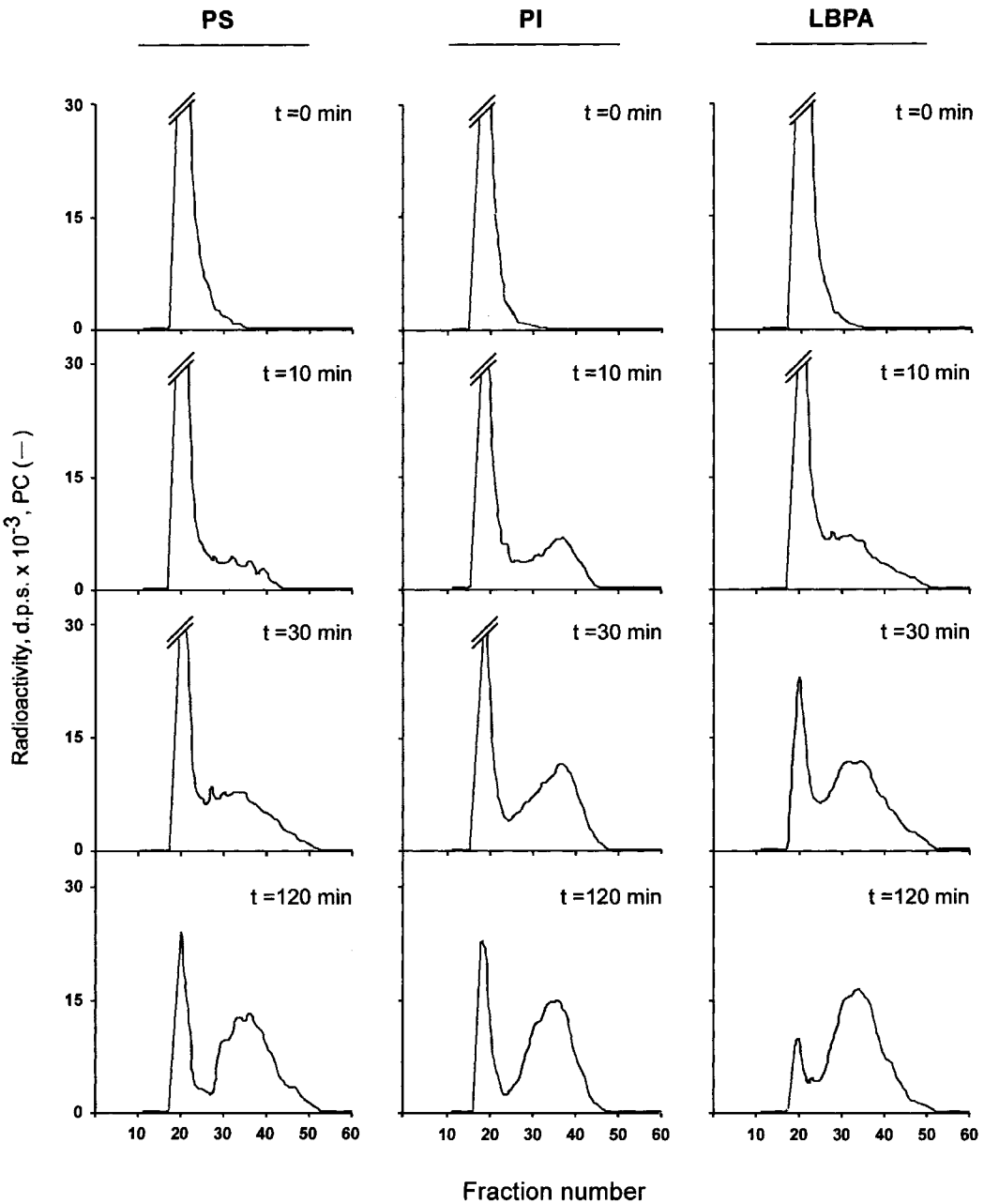

Figure 2 Kinetics of the Sap D-induced disruption of vesicles containing different anionic phospholipids

MLVs composed of PC/Chol/anionic phospholipid (65:25:10), containing trace amounts of [^{14}C]PC, were mixed with Sap D to yield a lipid/saposin molar ratio of 25:1. The anionic phospholipid was PS in the left panel, PI in the central panel and LBPA in the right panel. The samples were incubated in buffer A, pH 4.5, for the indicated times and then adjusted to pH 7.4 and analysed by gel filtration on Sepharose CL-4B columns as described in the Experimental section. The elution of the vesicles was monitored by the elution of PC given in terms of ^{14}C d.p.s.. The experiments when repeated at least three times gave similar elution profiles.

shows that Sap D was unable to induce the release of more than 5% of Calcein from Cer-containing LUVs, devoid of anionic phospholipids. When 25% of LBPA or PI or PS was also included into the vesicles, the leakage activity of Sap D increased dramatically. It is thus evident that Cer-containing lipid surfaces can be destabilized by Sap D only when anionic phospholipids are also incorporated into the membrane.

The effects of the three anionic phospholipids were markedly different, the inclusion of LBPA promoting the greatest destabilization (Figure 4). Interestingly, the initial extent of Sap D-induced leakage from vesicles containing both Cer and LBPA was higher than from vesicles containing LBPA alone (compare Figures 1 and 4). As shown in Table 1, the presence of Cer markedly increased the initial rate of Calcein release from LBPA-containing vesicles, while it inhibited the leakage from PI- or PS-containing membranes.

We have also compared the impact of high concentrations of Sap D on vesicles containing either LBPA alone or Cer and LBPA (Figure 5). At a lipid/saposin ratio of 50:1, MLVs containing only LBPA were partly disrupted, as indicated by gel-permeation analysis on a Sepharose CL-4B column (about 40% of lipids were eluted as a broad shoulder after the MLVs peak)

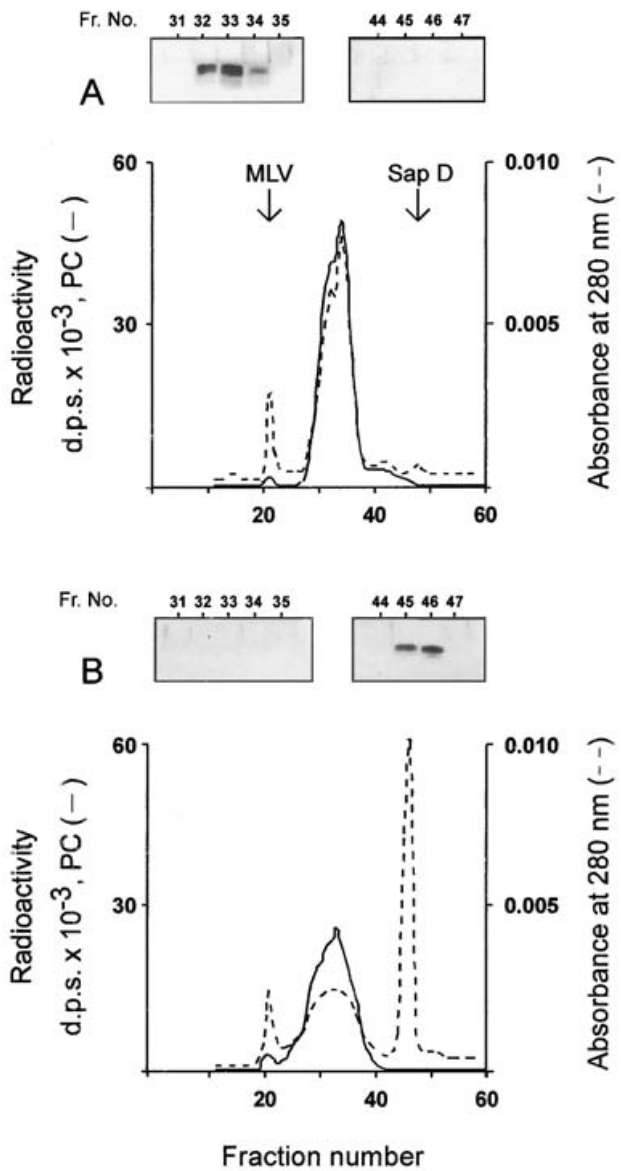

Figure 3 Reversibility of the Sap D binding to PS-containing vesicles

MLVs composed of PC/Chol/PS (65:25:10), containing trace amounts of [^{14}C]PC, were mixed with Sap D to yield a lipid/saposin molar ratio of 12.5:1 and incubated in buffer A, pH 4.5, for 2 h. Half of the sample was then analysed by gel filtration on a Superose 6 HR 10/30 prepacked column equilibrated and eluted with buffer A, pH 4.5 (**A**), while the other half of the sample was adjusted to pH 7.4 and loaded on the same column, equilibrated and eluted with buffer B, pH 7.4 (**B**) as described in the Experimental section. The relative absorbance at 280 nm was plotted as a function of elution volume (– – –). The elution of PC is given in terms of ^{14}C d.p.s. (——). The elution positions for MLVs and Sap D when eluted separately are indicated (arrows). The presence of Sap D in the fractions corresponding to the elution of lipids and of free Sap D was detected by electrophoretic analysis. The insets in (**A**) and (**B**) show the SDS/PAGE patterns of fractions eluted at pH 4.5 or 7.4 respectively. The experiments when repeated at least three times gave similar results.

(Figure 5A). Conversely, MLVs containing both LBPA and Cer were completely transformed into smaller particles that were eluted in a broad peak centred at fractions 29 and 30 (Figure 5B). Thus the presence of Cer further stimulates the disrupting activity of Sap D towards LBPA-containing membranes. By increasing the concentration of Sap D, the apparent size of the particles decreased, as judged from the shift of the elution peak from 29–30 (lipid/saposin molar ratio 50:1) to 34–35 (lipid/saposin molar

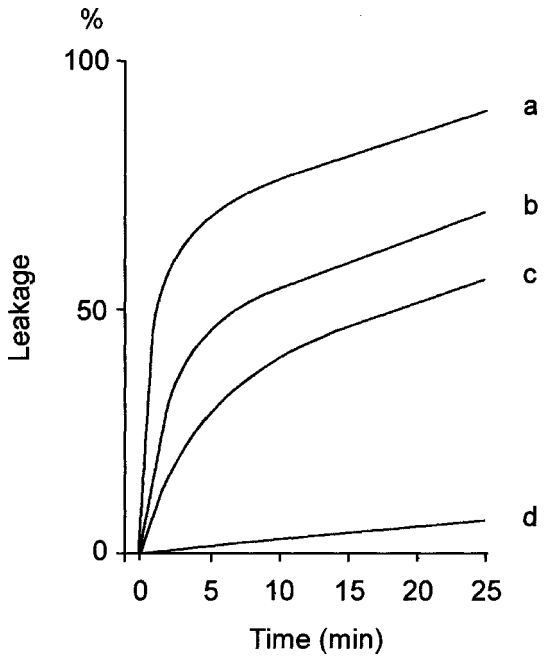

Figure 4 Effect of Cer on the Sap D-induced leakage of anionic-phospholipid-containing vesicles

Time course of the Sap D-induced release of Calcein entrapped in LUVs composed of PC/Chol/LBPA/Cer (40:25:25:10) (a), PC/Chol/PI/Cer (40:25:25:10) (b), PC/Chol/PS/Cer (40:25:25:10) (c) or PC/Chol/Cer (65:25:10) (d). The saposin was injected into a stirred cuvette thermostatically controlled at 37 °C, containing a 1 ml suspension of liposomes (75 μM total lipid) in buffer A, pH 4.5. The lipid/saposin molar ratio was 1000:1. The experiments when repeated at least three times gave similar results.

Table 1 Effect of Cer on the initial rate of the Sap D-induced leakage of anionic phospholipid-containing vesicles

The initial rate of the Sap D-induced leakage was determined from the tangent at time (t) = 0 to fluorescence curves such as those presented in Figures 1 and 4. The LUVs without Cer were composed of PC/Chol/anionic phospholipid (50:25:25). The LUVs with Cer were composed of PC/Chol/Cer/anionic phospholipid (40:25:10:25). The anionic phospholipids were LBPA or PI or PS as indicated. The Sap was injected into a stirred cuvette thermostatically controlled at 37 °C, containing a 1 ml suspension of liposomes (75 μM total lipid) in buffer A, pH 4.5. The lipid/saposin molar ratio was 1000:1. Results are means for at least three independent experiments.

	Initial leakage (%/s)	
Anionic phospholipid	− Cer	+ Cer
LBPA	1.0	1.7
PI	0.9	0.7
PS	0.9	0.4

ratio 25:1) (Figures 5B and 5C). The PC/Cer molar ratio in the small particles was the same as in the original MLVs (PC/Cer = 4), indicating that Cer is solubilized to the same extent as PC.

It has been previously reported that Sap D, as well as the other three saposins (Sap A, Sap B and Sap C) and prosaposin, has the capacity to bind, solubilize and transfer gangliosides particles [11]. We have investigated whether Sap D was able to solubilize a ganglioside such as G_{M1} inserted into phospholipid membranes. Figures 6(A) and 6(C) show that membranes composed of PC, Chol and G_{M1}, but devoid of anionic phospholipids, are minimally disrupted, even at high Sap D concentrations (3% at a lipid/saposin molar ratio of 25:1 and less than 10% at a ratio

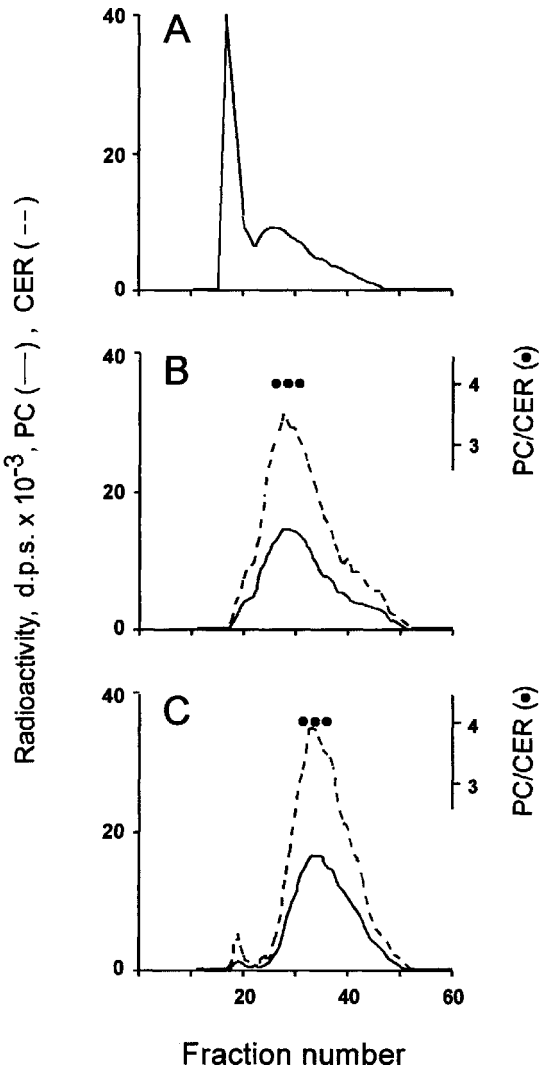

Figure 5 Effect of Cer on the Sap D-induced disruption of LBPA-containing vesicles

LBPA-containing MLVs with (**B** and **C**) or without (**A**) Cer were mixed with Sap D to yield a lipid/saposin molar ratio of 50:1 (**A** and **B**) or 25:1 (**C**). The compositions of MLVs were the following: (**A**) PC:Chol:LBPA (50:25:25), containing trace amounts of [^{14}C]PC; (**B**) and (**C**) PC/Chol/LBPA/Cer (40:25:25:10, containing trace amounts of [^{14}C]PC and [^{3}H]Cer. The samples were incubated in buffer A, pH 4.5, for 2 h, then adjusted to pH 7.4 and analysed by gel filtration on Sepharose CL-4B columns. The elution of PC (——) is given in terms of ^{14}C d.p.s. and the elution of Cer (– – –) in terms of ^{3}H d.p.s.. The molar ratios PC/Cer (●) for the fractions across the broad elution peaks are shown in (**B**) and (**C**). The experiments when repeated at least three times gave similar elution profiles.

of 12.5:1). Conversely, in the presence of an anionic phospholipid such as LBPA, the transformation of 80–90 % of the vesicles into small G_{M1}-containing particles was observed (Figures 6B and 6D). At a lipid/saposin molar ratio of 25:1 the particles were eluted as a peak centred around fraction 38, while at a molar ratio of 12.5:1, the peak was shifted towards lower molecular masses (maximum at fractions 42 and 43), confirming that the size of the particles depends on the saposin/lipid molar ratio (see Figure 5 and [18]). In the particles the PC/G_{M1} molar ratio (ranging from 3.7 to 3.5) was lower than in the original membranes (PC/G_{M1} = 4), indicating an enrichment in G_{M1}.

These results show that G_{M1}, as well as Cer, is solubilized by Sap D as part of phospholipid particles.

By comparing the solubilizing effects of Sap D on LBPA-containing vesicles in which either Cer or GM1 were included (see Figures 5 and 6), some differences can be noted: (a) at the same lipid/saposin molar ratio (25:1) the extent of MLV disruption in the presence of Cer is higher than in the presence of G_{M1}; (b) the G_{M1}-containing small particles are richer in G_{M1} than the original membranes, whereas the Cer-containing particles have the same Cer content.

DISCUSSION

The involvement of saposins in the enzymic degradation of sphingolipids has for a long time emphasized investigations reporting their possible interaction with sphingolipids or sphingolipid hydrolases [1–3]. During the past few years we have instead directed our attention on the interaction of saposins with phospholipid membranes and have thus uncovered for the first time the high affinity of Sap C and Sap D for anionic phospholipid-containing lipid surfaces [14–19]. Our findings, later confirmed by other groups [10,27], have recently acquired a special significance when it has been found that the late endosomes/lysosomes, where saposins are localized, contain a complex system of internal membranes rich in three anionic phospholipids, namely PI, PS and LBPA [21–22,28]. The distribution of phospholipids in these inner membranes varies significantly forming a mosaic of distinct lipid domains, where LBPA can account for up to 60–70 %, PI for up to 16 % and PS for up to 10 % of total phospholipids [22]. We have now investigated the capacity of Sap D to destabilize membranes containing either LBPA or PI or PS. A marked perturbation is observed in the presence of each of these three anionic phospholipids, the activity of Sap D being slightly higher towards LBPA-containing membranes. The great affinity of Sap D for LBPA, PI and PS strongly suggests that these lipids, clustering in the inner endosomal/lysosomal membranes, are the physiological target for Sap D.

At high Sap D concentrations, the saposin binding to anionic phospholipid-containing lipid surfaces results in the breakdown of the membrane to yield small particles enriched in anionic phospholipids [18]. It can be inferred that a high content of Sap D per vesicle destabilizes the membrane structure to such an extent as to lead to its disruption. The effects of Sap D on vesicles can be described in terms of Sap D segregating on to lipid surfaces of appropriate composition up to a critical lipid/protein molar ratio, followed by a marked decrease in the particle size. We have presently observed that the breakdown process occurs over a period of 30–60 min, the time being dependent on the type of anionic phospholipids included into the vesicles. Membrane-perturbing proteins usually alter lipid membrane organization in two steps: one is the binding of the protein to the membrane and the other is a change of its conformational or aggregational properties, resulting in membrane disruption [29]. The slow Sap D-induced breakdown of the vesicles might be due to a slow binding and/or to a rearrangement of the saposin into the membrane. The second hypothesis is more likely, since we found that Sap D binding to anionic phospholipid-containing membranes is very rapid [15].

We have previously shown that the binding, destabilizing and disrupting activity of Sap D is pH-dependent. In fact, acidic conditions mimicking the interior pH of lysosomes dramatically increase the hydrophobicity of Sap D and promote its association with membranes [15]. Acidification plays an essential role in regulating the function of proteins in the late endosomal/lysosomal compartment [30]. We have presently found that the Sap D association with lipid surfaces is reversible and that the saposin–lipid complexes formed in an acidic milieu are

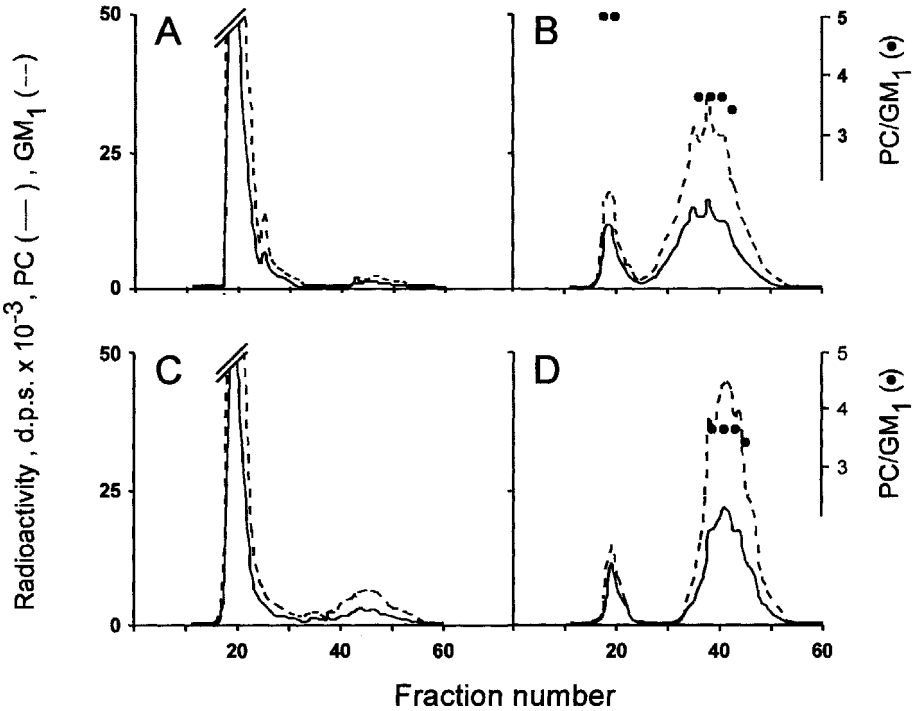

Figure 6 Effect of LBPA on the Sap D-induced disruption of G_{M1}-containing vesicles

G_{M1}-containing MLVs with (**B**, **D**) or without (**A**, **C**) LBPA were mixed with Sap D to yield a lipid/saposin molar ratio of 25:1 (**A** and **B**) or 12.5:1 (**C** and **D**). The compositions of MLVs were the following: (**A**) and (**C**) PC/Chol/GM1 (65:25:10), containing trace amounts of [^{14}C]PC and [^{3}H]G_{M1}; (**B**) and (**D**), PC/Chol/LBPA/G_{M1} (40:25:25:10), containing trace amounts of [^{14}C]PC and [^{3}H]G_{M1}. The samples were incubated in buffer A, pH 4.5, for 2 h, then adjusted to pH 7.4 and analysed by gel filtration on Sepharose CL-4B columns. The elution of PC (——) is given in terms of ^{14}C d.p.s. and the elution of G_{M1} (– – –) in terms of ^{3}H d.p.s.. The molar ratios PC/G_{M1} (●) for the fractions across the broad elution peaks are shown in (**B**) and (**D**). The experiments repeated at least three times gave similar elution profiles.

dissociated by an increase in pH. Since the luminal pH of the endosomal/lysosomal compartment is dynamic and can shift in the range 4.5–6.0 [30], the membrane association of Sap D may also be dynamic. Factors influencing the acidification inside the endocytic organelles can thus modulate the membrane-perturbing activity of Sap D.

A putative role of Sap D in Cer degradation has been proposed [9,10]. Sap D is able to stimulate the enzymic hydrolysis of Cer included in anionic phospholipid-containing membranes also *in vitro* [10]. The presently observed Sap D-induced perturbation of Cer containing membranes parallels the Sap D activation of the Cer degradation, both processes requiring the presence of anionic phospholipids, especially LBPA. This observation strongly suggests that activation of Cer hydrolysis might be ascribed to the structural impact of Sap D on the lipid surfaces. A number of studies have correlated the perturbation of membranes with the activation of membrane-bound enzymes [31–33]. For instance, we have previously shown that Sap C activation of the membrane-bound enzyme glucosylceramidase is correlated with Sap C-induced destabilization of anionic phospholipid-containing membranes [19,20]. It can be envisaged that the high perturbing activity of Sap D towards anionic-phospholipid-containing surfaces affects the Cer organization in the lipid surface, modulating in this way the meeting of Cer with membrane-bound ceramidase.

Another factor that might explain the Sap D-promoted degradation of Cer in the presence of anionic phospholipids is the Sap D ability to solubilize large vesicles with formation of small particles having the same Cer content as the original vesicles (see Figure 5). It is well known that the dimension of the substrate-containing vesicles has a great influence on the activities of membrane-associated enzymes such as ceramidase [10,32]. Also the lysosomal membrane-bound glucosylceramidase is able to degrade glucosylceramide inserted in small unilamellar vesicles at a rate 7–8-fold higher than glucosylceramide inserted in large vesicles [14]. Most likely, the loose packing of the lipids in the outer surface of small vesicles favours the enzyme interaction with the lipid surface.

It was presently observed that the inclusion of Cer stimulates the Sap D-perturbing activity towards LBPA-containing vesicles, while it inhibits that towards PI- or PS-containing membranes. The inhibition may be ascribed to the increased rigidity of the membrane after the inclusion of Cer. On the other hand, the fact that Cer increases the susceptibility of the LBPA-containing vesicles to the Sap D action indicates that the interaction between Cer and LBPA differs from that with PI or PS. The interactions between Cer and phospholipids have been extensively studied [34–36]. Recent studies have reported that the lack of bulky headgroup in the Cer molecule allows for closer chain interactions and the formation of complexes between Cer and phospholipid molecules, which laterally separate from the bulk lipids [31]. The increased Sap D affinity towards lipid surfaces containing Cer and LBPA suggests that specific complexes between these two lipids might form domains that favour the Sap D interaction with membranes.

Previous investigators have claimed that saposins, as well as prosaposin, bind and solubilize gangliosides and neutral sphingolipids [11,12]. It was assumed that saposins directly bound sphingolipids, while the possible influence on binding of other lipid components of the membrane was not considered. Our present work shows that the Sap D-induced solubilization of a ganglioside such as G_{M1} or of a neutral sphingolipid such as

Cer, requires the presence of anionic phospholipids. In other words, Sap D has poor affinity towards sphingolipids and its impact on sphingolipid-containing membranes is governed by the Sap D interaction with anionic phospholipids. Moreover, under our experimental conditions, mimicking the environment of the endosomal/lysosomal compartment (low pH values and presence of LBPA-rich membranes), Cer and G_{M1} are not solubilized as such, but as constituents of small phospholipid particles. We thus believe that previous results and conclusions on the binding and solubilization of sphingolipids by saposins must be reconsidered in view of the preferential interaction of at least two saposins, Sap C and Sap D, with anionic phospholipids.

Our past and present work has extensively documented the destabilizing and solubilizing properties of Sap D. Collectively, our results offer evidence that the affinity towards anionic phospholipids plays a key role in governing the Sap D impact on lipid surfaces. Also the Sap D effects on sphingolipid-containing membranes are mediated by the binding of Sap D to anionic phospholipids. This interaction most likely underlies most of the proposed biological functions of Sap D, which include the promotion of Cer degradation and the solubilization and transport of gangliosides. Considering that Sap D is localized in the lysosomal/endosomal compartment that contains internal membranes very rich in anionic phospholipids, our observations have broad implications for possible effects of Sap D on the structure and function of these membranes, either in the presence or in the absence of sphingolipids.

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